The use of silver nitrate for the identification of spermatozoon structure in selected mammals

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Andraszek, K. and Smalec, E. 2011. **The use of silver nitrate for the identification of spermatozoon structure in selected mammals**. Can. J. Anim. Sci. **91**: 239–246. The spermatozoon is one of the most diversified cell types, and the chromatin of the haploid spermatozoon genome is essentially different from that of the somatic cell as regards its chemical composition, structure and function. Although the structure of spermatozoon chromatin has crucial importance for fertilization and embryo development, standard staining techniques are still predominantly used for identifying semen quality and the assessment of spermatozoa is most often limited to detecting irregularities in their morphological structure. The aim of the present research was to evaluate the usefulness of silver nitrate staining for assessing spermatozoon morphology and identifying spermatozoon structure. Spermatozoa isolated from testes and semen were examined. Silver nitrate staining made it possible to identify many significant details of the morphological structure of the spermatozoon and could be successfully employed in sperm morphology assessments.

Key words: Spermatozoa, testicle, spermatogenesis

Andraszek, K. et Smalec, E. 2011. **Utilisation du nitrate d'argent pour étudier la structure des spermatozoïdes chez certains mammifères**. Can. J. Anim. Sci. **91**: 239–246. Le spermatozoïde est l'une des cellules les plus diversifiées qui soient et la chromatine du spermatozoïde haploïde diffère essentiellement de celle de la cellule somatique pour ce qui est de sa composition chimique, de sa structure et de sa fonction. Bien que la structure de la chromatine dans le spermatozoïde joue un rôle capital dans la fécondation et le développement de l'embryon, les techniques de coloration usuelles servent encore principalement à identifier la qualité du sperme et l'évaluation des spermatozoïdes se limite souvent à la détection des anomalies morphologiques. Les chercheurs voulaient établir l'utilité de la coloration au nitrate d'argent pour étudier la morphologie et préciser la structure des spermatozoïdes. Ils ont examiné des spermatozoïdes isolés des testicules et du sperme. La coloration au nitrate d'argent permet d'identifier de nombreux détails morphologiques importants dans la structure des spermatozoïdes et on pourrait s'en servir pour évaluer la morphologie du sperme.

Mots clés: Spermatozoïdes, les testicules, la spermatogenèse

The development of microscope technology and in vivo analysis methodology that has taken place over the past 10 yr has led to significant advances in the exploration of endonuclear genome setup of the diploid somatic cell. One of the most intriguing properties of the diploid cell nucleus is its capacity to become transformed into a haploid sperm nucleus (Claussen 2005; Dehghani et al. 2005; Foster and Bridger 2005). This transformation is primarily caused by the replacement of histones with protamines, as a result of which sperm chromatin DNA is no longer superhelically twisted. Following sperm and egg cell fusion and prior to the first mitotic division of the zygote, an opposite process takes place. Thus, the connection between the conformation of somatic chromatin and spermatozoon-related conformation is manifested in the capacity for bidirectional transformation of both structural types of chromatin. The purpose of such radical conformational change and the resultant sperm chromatin structure are not entirely clear (Cremer et al. 2004; Zalenskaya and Zalensky 2004; Ainsworth 2005; Claussen 2005; Kimmins and Sassone-Corsi 2005; Mudrak et al. 2005; Shaman and Ward 2006).

The chromatin structure of a mature spermatozoon is associated with the reversible "dormant state" of the genome in which no replication or transcription occurs. In this regard, the sperm nucleus offers a unique model for comparing active genome organization, as opposed to inactive genome setup. During the process in question, the pores in the nuclear envelope disappear, the nucleolus becomes invisible and chromatin undergoes intensive condensation, as a result of which the transcription capacity of the nucleus is blocked. In comparison with somatic cell chromatin, this chromatin does not exhibit any differences only at the level of the double DNA helix (Szczygieł and Kurpisz 1996; Braun 2001; Cremer et al. 2004; Kimmins and Sassone-Corsi 2005; Shaman and Ward 2006).

Chromatin reorganisation is closely linked to chemical changes taking place in the nucleus structure. H1 histones, present in chromatin, become temporarily replaced with protamines inside spermatids, as a result of which chromatin loses its original nucleosomal structure. Owing to their bisulphate bonds, protamines, present in cysteine residues, condense and stabilise chromatin.

Due to this condensation, chromatin-packed DNA is more resistant to mutation and chemical damage, which are imminent during the journey of a spermatozoon towards the egg cell. During chromatin condensation and, basically, simultaneously with it, there occurs a fundamental change in the nucleus shape. According to one of the hypotheses, nucleus shape can be genetically determined by genetically controlled packing patterns of DNA and proteins during chromatin condensation (Kleene 1993; Penttillä 1995; Strzeżek 1998; Braun 2001; Cremer et al. 2004; Ward and Ward 2004; Mudrak et al. 2005; Conner and Barratt 2006; Shaman and Ward 2006).

The part closer to the tail is the midpiece. Inside the midpiece, there are elongated mitochondria arranged in a single cord. The number of mitochondria in the midpiece and the number of twists of their spiral is specific to particular mammal species. Mitochondria occupy 80% of the midpiece surface and are arranged in a spiral, which ensures an optimal amount of ATP, indispensable for sperm mobility. Moreover, the energy generated by mitochondria is used for the active transport of substrates through membranes, sperm hyperactivation and acrosomal reaction, which are processes necessary for fertilization. Thus, these organelles become essential structures that define the potential of spermatozoa for egg cell fertilization. Damage to sperm mitochondria is also not irrelevant to a dividing fertilized egg cell. In adequate conditions, it is capable of recognising sperm mitochondria as foreign elements and removing them through mitochondrial protein ubiquitination, which involves the removal of paternal mtDNA. Mitochondrial damage can disturb the process of mitochondrial degradation, which, in turn, can lead to apoptosis of the dividing blastomeres and the subsequent decay of the pre-implantation embryo (Eddy and O'brien 1994; Chandler et al. 2000; Paasch et al. 2004; Piasecka 2004a, b; Martinez-Pastor et al. 2008).

The light microscope makes it possible to identify the head with the acrosome at the front, the midpiece and the main part of the tail in mammalian and human spermatozoa. In most cases, the sperm nucleus is flattened and its size as well as the shape of the whole head are species-specific (Bielańska-Osuchowska and Sysa 1998; Strzeżek 1998; Sutovsky and Manandhar 2006; Veeramachaneni 2006).

However, due to their size and compact organization, the structure of mammalian spermatozoa cannot be analysed in detail using a light microscope. Advanced semen cryopreservation methods require an assessment of the influence of this process and its various variants on sperm structure and the maintenance of physiological functions of spermatozoa. In vitro fertilization has intensified research on changes in sperm morphology and ultrastructure. A separate group of issues have been studies explaining infertility of men and farm animal males. The results of the above research have significantly contributed to developing the in vitro fertilization method on a large scale. However, they have not produced a lot of new data relating to sperm ultrastructure.

The aim of this paper is to evaluate the usefulness of silver nitrate staining for assessing spermatozoon morphology and identifying spermatozoon structure.

MATERIAL AND METHODS

The analysis concerned spermatozoa isolated from the testes and semen of domestic pigs and cattle. Since the studies were meant to determine the usefulness of silver nitrate ($AgNO_3$) staining for the identification of particular elements of spermatozoon structure, no detailed morphological analysis of the spermatozoa was performed and the defects of the spermatozoa contained in the semen were also not assessed.

The spermatozoa were isolated from the testes according to the methodology described by Evans et al. (1964), which is routinely applied for isolating meiotic chromosomes. The fixed cells were suspended in a small amount of fresh Carnoy fixer, spread over degreased and refrigerated slides and dried at room temperature.

Preparations of the fresh and diluted semen were made using the smear technique. Preparations of the semen in Carnoy fixer were made experimentally. A sample of the undiluted semen (1 mL) was suspended in 7 mL of fresh Carnoy's fixer and then the suspension was centrifuged for 5 min at 800 revolutions per minute. The fixing procedure was repeated threefold. The fixed sperm suspension was spread over slides and dried at room temperature.

The preparations were stained with the solution ($AgNO_3$) following Howell and Black's (1980) methodology, routinely used for the identification of nucleolus organizer regions in mitotic chromosomes and nucleoli in the prophase of the first meiotic division. A 50% $AgNO_3$ solution and a colloidal gelatin solution were applied on the 1-wk-old preparations. The preparations were covered with a cover glass and incubated for 15–20 min in an incubator at 60°C, in complete humidity. After the preparations turned gold in colour, the chemical reaction was interrupted and the preparation rinsed several times with distilled water. The preparations were dried at room temperature.

The dyed preparations were analysed using the Olympus BX50 light microscope with a 100-fold magnification. The extent of staining and the possibility of identifying the greatest possible number of morphological details in the structure of a spermatozoon were assessed. The digital analysis of the preparations was performed using the Multiscan image analysis system from Computer Scanning Systems.

RESULTS

Figures 8–10 show sample spermatozoa of the boar and the bull, isolated from the testes as well as fixed and fresh semen.

Boar Spermatozoon Structure

Figure 1 depicts sample boar spermatozoa isolated from the testis. Within a spermatozoon, it is possible to distinguish the head (A) with the acrosomal part (closed arrow) and the distal postacrosomal region (cap) (open arrow). Within a spermatozoon, it is possible to distinguish the light midpiece (B) and the dark remaining part of the tail (C) (Fig. 8a). The spermatozoon in Fig. 8b has a dark nucleolus (arrow). Moreover, the spermatozoa have characteristic "collars" (arrowhead).

Boar spermatozoa isolated from the fixed semen no longer have the characteristic "collar" after staining with silver nitrate (Fig. 2a, b). The respective elements of spermatozoon structure: the head (A), light midpiece (B) and the rest of the tail (C) are easily discernible. Within the spermatozoon head, it is possible to distinguish the light acrosomal part (closed arrow) and the dark distal postacrosomal region (cap) (open arrow).

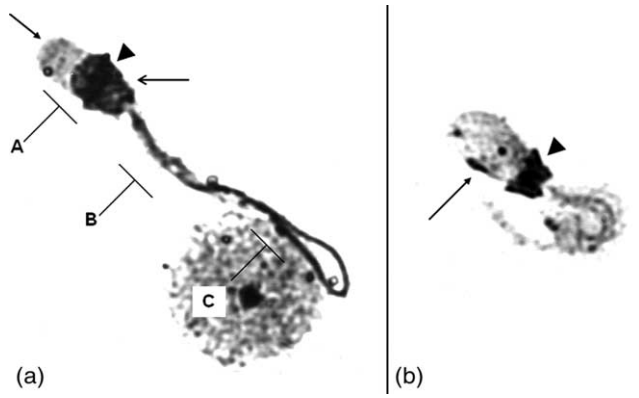

Fig. 1. Boar spermatozoa isolated from the testis – $AgNO_3$ staining.

Staining with silver nitrate also makes it possible to observe the course of the acrosomal reaction (Fig. 2 c, d) (arrowhead).

After $AgNO_3$ staining, the same morphological details were observed in the boar spermatozoa isolated from the fresh semen as from the fixed semen. The head (A), the midpiece – lighter than the rest of the tail (B) and the dark remaining part of the tail (C) were identified. The head of the spermatozoon was clearly divided into the light acrosomal part (closed arrow) and the dark distal one (open arrow) (Fig. 3).

Bull Spermatozoon Structure

In the bull spermatozoa isolated from the testis and stained with silver nitrate, the following elements were identified: the dark head (A), the light midpiece (B) and the dark remainder of the tail (C). Within the spermatozoon head, it is possible to distinguish the light acrosomal part (closed arrow) and the dark distal postacrosomal region (cap) (open arrow). All the spermatozoa presented had the characteristic dark "collar" (arrowhead) (Fig. 4).

Figure 5 depicts spermatozoa with extremely kinked tails. This is a primary defect that results in diminished fertility of an animal. In morphological assessments, this entanglement of the tail is called the "Dag defect" (Morstin 1996). Such a spermatozoon will not develop a proper movement apparatus. In spite of the kinked tail, it is possible to make out the light midpiece (closed arrow) and the dark remainder of the tail (open arrow). One can also see the vividly coloured distal cap with the characteristic "collar" (arrowhead).

Bull spermatozoa obtained from both fixed (Fig. 6) and fresh semen (Fig. 7) were characterised by the same morphological structure. In the spermatozoa, we distinguished the head (A) within which the light

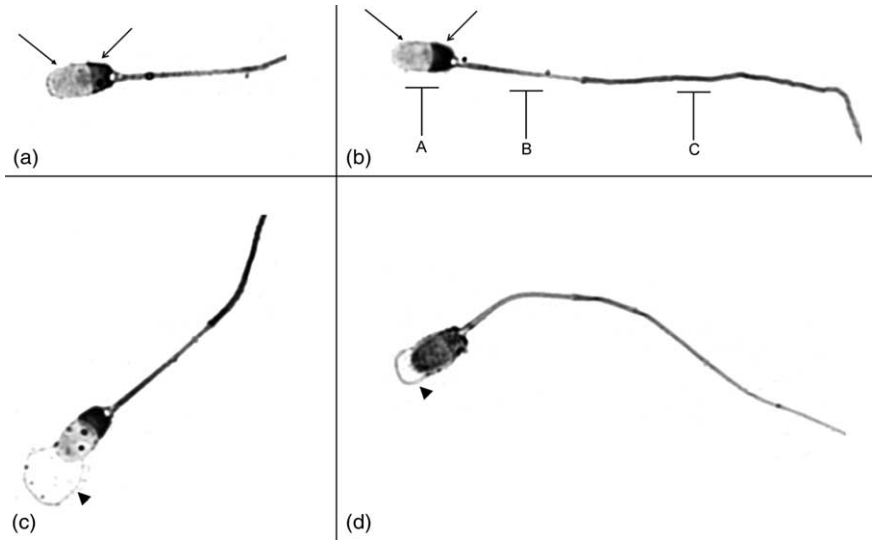

Fig. 2. Boar spermatozoa isolated from the fixed semen – $AgNO_3$ staining.

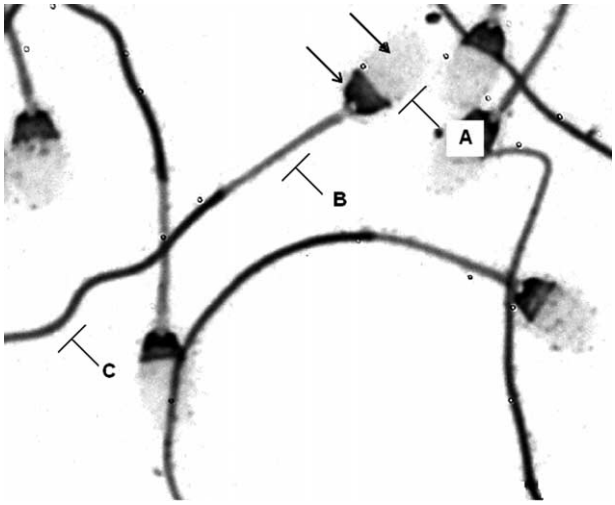

Fig. 3. Boar spermatozoa isolated from the fresh semen – $AgNO_3$ staining.

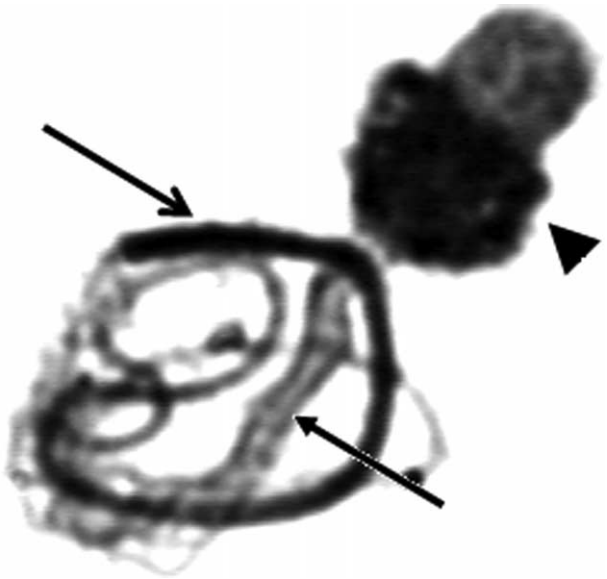

Fig. 5. Spermatozoa with the Dag defect, isolated from a bull testis; $AgNO_3$ staining.

acrosomal (closed arrow) and the dark distal part (open arrow) were identified. Moreover, staining with silver nitrate differentiated the spermatozoon tail into the lighter midpiece (B) and the dark remainder of the tail (C).

DISCUSSION

Over the past few years, spermatozoon structure has been predominantly dealt with from the point of view of possible applications in medicine, veterinary science and animal husbandry. However, sperm assessments are still limited to identifying irregularities in spermatozoon structure. Most spermatozoon defects that limit sperm capacity for fertilization result from irregularities in spermatogenesis. Hence, these changes are of a molecular and cytogenetic nature.

Part of the results, especially those relating to the spermatozoa isolated from the testes, have no frame of reference in relevant literature. Where reference was possible, the results were juxtaposed with other authors' research.

Silver nitrate is an alkaline stain used for the identification of acidic chromatin proteins, nucleolus organizers and nucleoli (Goodpasture and Bloom 1975; Howell and Black 1980; Andraszek and Smalec 2007; Andraszek et al. 2009). Routine assessments of spermatozoon morphology in cattle and pigs most often involve the use of the eosin–gentian violet complex or the eosin–nigrosin complex. Eosin is an acidic stain used for the identification of alkaline molecules. Eosin–gentian violet staining is a routine technique for evaluating the morphology of farm animal semen, used, among others, for assessing the morphology of getter semen at Animal Breeding and Insemination Stations (Kondracki et al. 2006; Wysokińska et al. 2008). Apart from facilitating assessments of spermatozoon morphology, eosin–nigrosin staining also makes it possible to identify living and dead spermatozoa (Swanson and Bearden 1951; Sprecher and Coe 1996; Zambelli and Cunto 2006; Freneau et al. 2009). In addition, eosin–nigrosin staining is recommended by the WHO for describing human sperm morphology (Björndahl et al. 2003, 2004).

The use of silver nitrate in the research discussed here could, according to the authors, reveal morphological

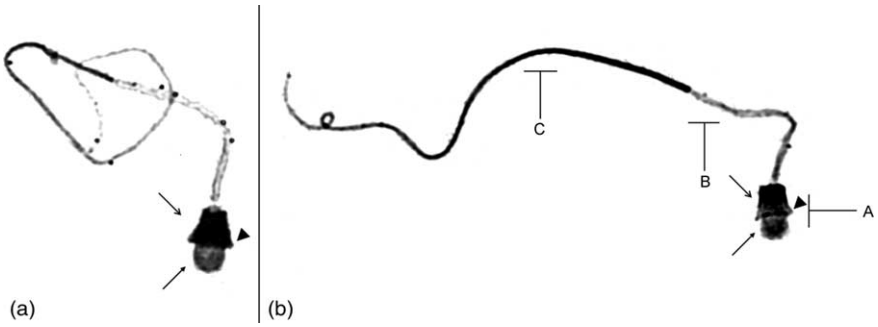

Fig. 4. Bull spermatozoa isolated from the testis – $AgNO_3$ staining.

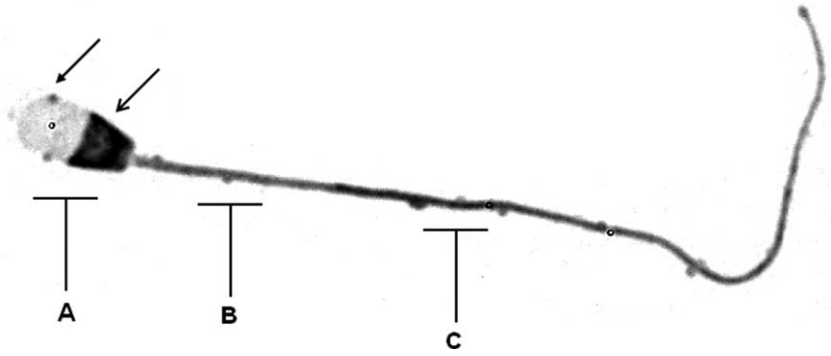

Fig. 6. Bull spermatozoa isolated from the fixed semen – $AgNO_3$ staining.

details of spermatozoa that are invisible after applying acidic stains. Figures 8 and 9 present the differentiated structure of spermatozoa isolated from the fresh semen of the boar and the bull, respectively, evident after staining with silver nitrate and the eosin-gentian violet complex.

Another aspect of the research was the comparison of spermatozoa isolated from the testes with spermatozoa from the semen, and the determination of the usefulness of the semen preparations fixed in the Carnoy solution for sperm morphology analysis. No changes in morphological structure were found in the spermatozoa from the semen fixed in the Carnoy solution. The same structural details were identified in them as in the spermatozoa isolated from the fresh semen. It was concluded that the Carnoy solution can be successfully used for fixing spermatozoa intended for morphology assessments, particularly if the analysis cannot be performed on fresh preparations, or it is necessary to repeat the experiment.

Sperm head proteins are alkaline. Therefore, after applying silver nitrate, the acrosomal part of the head was less intensively stained than the distal part. Silver nitrate staining shows that sperm nucleus chromatin has a different composition in the acrosomal part than in the distal one, which contains acidic proteins, and the nucleolus that positively reacts with silver salts. After staining with silver nitrate, the spermatozoa isolated from the testes were characterized by dark "collars" in the distal part of the head. The "collars" were not observed in the mature spermatozoa. This phenomenon could be accounted for by the course of spermiogenesis. When the sperm head is formed out of the spematid nucleus, the RNA is removed from the nucleus. After the acrosomal vesicle is fully developed, the spermatid assumes an elongated shape, while the cytoplasm gradually becomes separated from the nucleus. The dark silver deposit in the form of a "collar" can be the result of a reaction between silver and the RNA, removed from the nucleus and present in the cytoplasm.

What is of special interest are the differences in the staining of the sperm heads, which can stem from the changing composition of the nuclear proteins during spermatogenesis. Chromatin of the still diploid spermatocyte of the first order has a structure that is typical of a somatic cell. In this phase, one can observe relatively large meiotic chromosomes. In a mature spermatozoon, histones are replaced with protamines. The elimination of histones gradually progresses throughout spermatogenesis. As a result of replacing histones with protamines

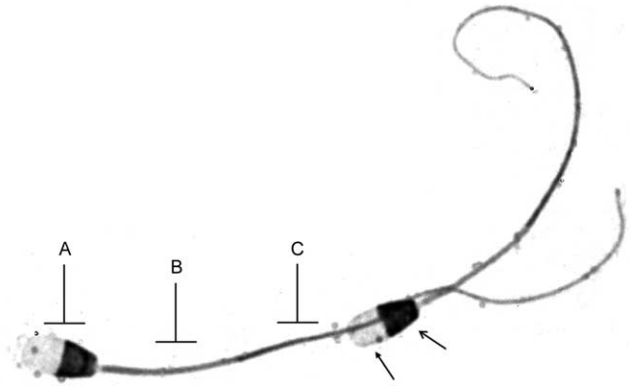

Fig. 7. Bull spermatozoa isolated from the fresh semen – $AgNO_3$ staining.

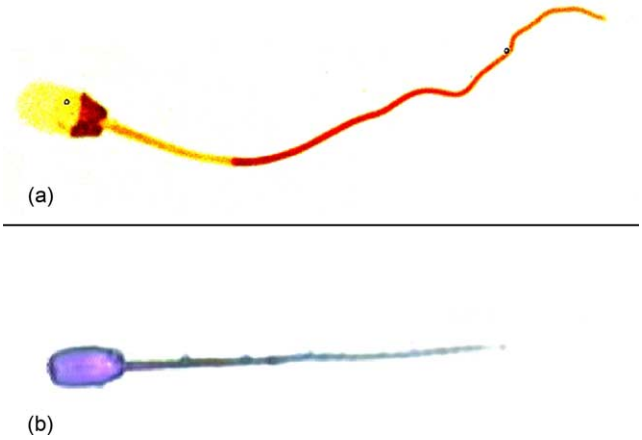

Fig. 8. Boar spermatozoa stained with $AgNO_3$ (a) and eosin (b).

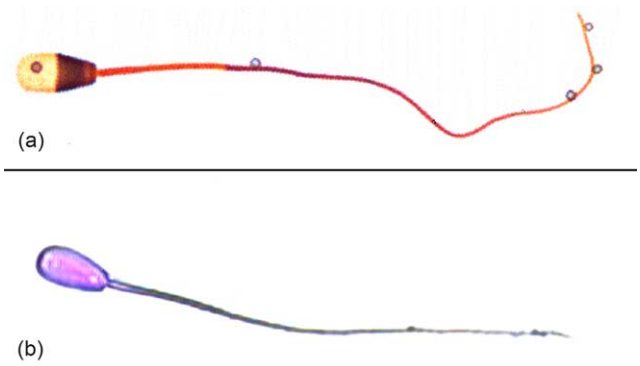

Fig. 9. Bull spermatozoa stained with $AgNO_3$ (a) and eosin (b).

that are half their size, the organisation of sperm chromatin completely changes as compared with spermatocyte chromatin (Bellev et al. 1988; Lee et al. 1995; Bench et al. 1996; Braun 2001; Meistrich et al. 2003; Mudrak et al. 2005; Conner and Barratt 2006). Figure 10 presents the difference in chromatin condensation in a boar spermatocyte of the first order and a boar spermatozoon.

The changes that occur at the level of chromatin and result from the replacement of one type of protein with another one with different chemical properties produce the differentiated pattern of sperm head staining in the spermatozoa isolated from the testes as compared with the mature spermatozoa.

The importance of fundamental proteins for stabilizing the DNA structure has been noted in recent years. The use of cytochemical methods makes it possible to determine changes in the nature of the histone-like proteins present in spermatids that turn into stable protamine proteins in spermatozoa. In certain cases of

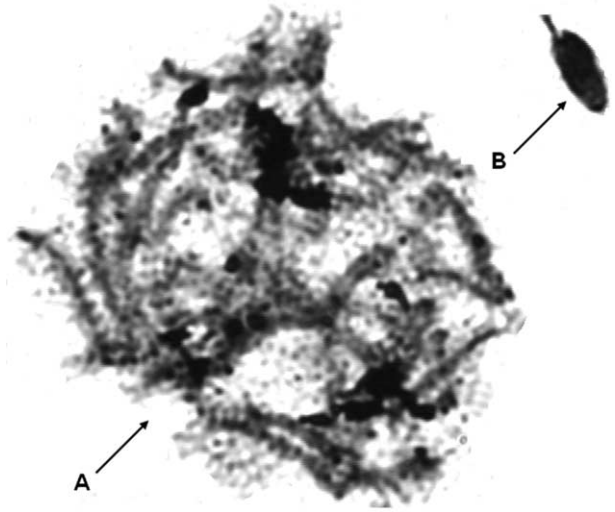

Fig. 10. Different chromatin condensation in a primary spermatocyte (A) and spermatozoon (B) of the boar (own research, unpublished).

infertility in bulls, the direct reason lies in irregularities in the transformation of proteins during spermiogenesis, not in elevated or reduced DNA levels. Moreover, disruptions of the nucleoprotein structure can be regarded as possible markers of the biological value of spermatozoa (Bench et al. 1996; Strzeżek 1998; Meistrich et al. 2003; Martins et al. 2004; Martianov et al. 2005).

Another element of sperm structure that was considered during the analysis of the preparations was the midpiece. Eighty percent of the midpiece volume is constituted by mitochondria, which ensure an optimal amount of energy necessary for sperm movement (Sutovsky and Manandhar 2006). If mitochondrial defects are present in numerous spermatozoa, they lead to reduced fertility or complete infertility of an individual. In the case of humans, identification of mitochondrial malfunctions is considered a necessary test (Paasch et al. 2004; Piasecka 2004a, b).

Mitochondrial lesions are one of the most frequent sperm defects. Mitochondrial defects involve both morphological irregularities of these organelles and malfunctions. These are often molecular and genetic disturbances, which, in many cases, lead to the apoptosis of spermatogenic cells. In addition, abnormal mitochondria have influence on the dividing zygote. They may not be recognised by the egg cell, resulting in a miscarriage (Baccetti 1984; Kleene 1993; Piasecka et al. 2003; Paasch et al. 2004; Piasecka 2004a, b; Martinez-Pastor et al. 2008). Considering such diverse defects, precise morphological and functional diagnostics of sperm mitochondria should complement routine semen diagnostics in cases of infertility.

Advances in biology, molecular genetics and technology make it possible to discover more and more diverse aspects of sperm structure. In turn, such knowledge facilitates the detection and understanding of the reasons for spermatozoon defects, which, in many cases, are related to changes in nuclear and mitochondrial DNA, and result in spermatozoa with a modified morphological structure. Silver nitrate staining is a simple and cheap technique that produces quick results. Silver nitrate makes it possible to clearly identify the differentiated structure of the sperm head and midpiece. Therefore, it can successfully complement routine techniques for assessing sperm morphology or be used as an independent technique.

Ainsworth, C. 2005. The secret life of sperm. Nature **436**: 770–771.

Andraszek, K. and Smalec, E. 2007. Number and size of nucleoli in the spermatocytes of European domestic goose (*Anser anser*). Arch. Geflügelkd. **71**: 237–240.

Andraszek, K., Horoszewicz, E. and Smalec, E. 2009. Nucleolar organizer regions, satellite associations and nucleoli of goat cells (*Capra hircus*). Arch. Tierz. **52**: 177–186.

Baccetti, B. 1984. Human spermatozoon. Pages 110–126 *in* J. Blercom and P. M. Motta, eds. Ultrastructure of reproduction. Nijhoff, The Hague, the Netherlands.

Bellve, A., McKay, D., Renaux, B. S. and Dixon, G. H. 1988. Purification and characterization of mouse protamines P1 and P2. Amino acid sequence of P2. Biochemistry **27**: 2890–2897.

Bench, G. S., Fitz, A. M., Cotrzett, M. H., Morse, D. H. and Balhorm, R. 1996. DNA and total protamine masses in individual sperm from fertile mammalian subjects. Cytometry **23**: 263–271.

Bielańska-Osuchowska, Z. and Sysa, P. 1998. Ultrastruktura plemnika ssaka. Pages 77–98 *in* A. Łukaszczyk, B. Bilińska, J. Kawiak and Z. Bielańska-Osuchowska, eds. Ultrastruktura i funkcja komórki, mechanizmy regulujące spermatogenezę. Państwowe Wydawnictwo Naukowe PWN, Warszawa, Poland.

Björndahl, L., Söderlund, I. and Kvist, U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum. Reprod. **18**: 813–816.

Björndahl, L., Söderlund, I., Johansson, S., Mohammadieh, M., Pourian, M. R. and Kvist, U. 2004. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. J. Androl. **25**: 671–678.

Braun, R. E. 2001. Packaging paternal chromosomes with protamine. Nat. Genet. **28**: 10–12.

Chandler, J. E., Harrison, C. M. and Canal, A. M. 2000. Spermatozoal methylene blue reduction: an indicator of mitochondrial function and its correlation with motility. Theriogenology **15**: 261–271.

Claussen, U. 2005. Chromosomics. Cytogenet. Genome Res. **111**: 101–106.

Conner, S. J. and Barratt, Ch. L. R. 2006. Genomic and proteomic approaches to defining sperm production and function. Pages 49–71 *in* Ch. De Jonge and Ch. Barratt, eds. The sperm cell. Production, maturation, fertilization, regeneration. Cambridge University Press, Cambridge, UK.

Cremer, T., Kupper, K., Dietzel, S. and Fakan, S. 2004. Higher order chromatin architecture in the cell nucleus: on the way from structure to function. Biol. Cell. **96**: 555–567.

Dehghani, H., Dellaire, G. and Bazett-Jones, D. P. 2005. Organization of chromatin in the interphase mammalian cell. Micron **36**: 95–108.

Eddy, E. M. and O'Brien, D. A. 1994. The spermatozoon. Pages 29–77 *in* E. Knobil and J. D. Neill, eds. The physiology of reproduction. Raven Press, Ltd., New York, NY.

Evans, E. P., Breckon, G. and Ford, C. E. 1964. An airdrying method for meiotic preparation from mammalian testes. Cytogenetics **3**: 295–298.

Foster, H. A. and Bridger, J. M. 2005. The genome and the nucleus: a marriage made by evolution. genome organization and nuclear architecture. Chromosoma **114**: 212–229.

Freneau, G. E., Chenoweth, P. J., Ellis, R. and Rupp, G. 2010. Sperm morphology of beef bulls evaluated by two different methods. Anim. Reprod. Sci. **118**: 176–181.

Goodpasture, C. and Bloom, S. E. 1975. Visualization of nucleolar organiser regions in mammalian chromosomes using silver staining. Chromosoma **53**: 37–50.

Howell, W. M. and Black, D. A. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experentia **36**: 1014–1015.

Kimmins, S. and Sassone-Corsi, P. 2005. Chromatin remodelling and epigenetic features of germ cells. Nature **434**: 583–589.

Kleene, K. C. 1993. Multiple controls over the efficiency of translation of the mRNAs encoding transition proteins, protamines, and the mitochondrial capsule selenoprotein in late spermatids in mice. Dev. Biol. **159**: 720–731.

Kondracki, S., Banaszewska, D., Wysokińska, A. and Chomicz, J. 2006. Sperm morphology of cattle and domestic pigs. Reprod. Biol. **6**: 99–104.

Lewis, J. D., Sapera, N., Song, Y., Zamora, M. J., Chiva, M. and Ausio, J. 2004. Histone H1 and the origin of protamines. Proc. Natl. Acad. Sci. USA **101**: 148–152.

Martianov, I., Brancorsini, S., Catena, R., Gansmuller, A., Kotaja, N., Parvinen, M., Sassone-Corsi, P. and Davidson, I. 2005. Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. Proc. Natl. Acad. Sci. USA **102**: 2808–2813.

Martinez-Pastor, F., Fernández-Santos, M. R., del Olmo, E., Domínguez-Rebolledo, A. E., Esteso, M. C., Montoro, V. and Garde, J. J. 2008. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod. Fertil. Dev. **20**: 547–556.

Martins, R. P., Ostermeier, G. C. and Krawetz, S. A. 2004. Nuclear matrix interactions at the human protamine domain: a working model of potentiation. J. Biol. Chem. **279**: 51862–51868.

Meistrich, M. L., Mohapatra, B., Shirley, C. R. and Zhao, M. 2003. Roles of transition nuclear proteins in spermiogenesis. Chromosoma **111**: 483–488.

Morstin, J. 1996. Spermatogeneza. W: Andrologia. Red. Wierzbowski S. Wydawnictwo PLATAN – Kryspinów, Kraków.

Mudrak, O., Tomilin, N. and Zalensky, A. O. 2005. Chromosome architecture in the decondensing human sperm nucleus. J. Cell Sci. **118**: 4541–4550.

Paasch, U., Grunewald, S., Dathe, S. and Glander, H. J. 2004. Mitochondria of human spermatozoa are preferentially susceptible to apoptosis. Ann. N. Y. Acad. Sci. **1030**: 403–409.

Penttilä, T. L., Yuan, L., Mali, P., Höög, C. and Pervinen, M. 1995. Haploid gene expression: temporal onset and storage patterns of 13 novel transcripts during rat and mouse spermatogenesis. Biol. Reprod. **53**: 499–510.

Piasecka, M. 2004a. Morfologia i funkcja mitochondriów plemnika a męska płodność. Prawidłowa morfologia i funkcja wstawki plemnika. Post. Biol. Kom. **31**: 489–516.

Piasecka, M. 2004b. Morfologia i funkcja mitochondriów plemnika a męska płodność. Zaburzenia morfologiczno-funkcjonalne mitochondriów wstawki plemnika. Post. Biol. Kom. **31**: 517–541.

Shaman, J. A. and Ward, W. S. 2006. Sperm chromatid stability and susceptibility to damage in relation to its structure. Pages 31–49 *in* Ch. De Jonge and Ch. Barratt, eds. The sperm cell. Production, maturation, fertilization, regeneration. Cambridge University Press, Cambridge, UK.

Sprecher, D. J. and Coe, P. H. 1996. Differences in bull spermiograms using eosin-nigrosin stain, feulgen stain, and phase contrast microscopy methods. Theriogenology **45**: 757–764.

Strzezek, J. 1998. Fizjologia i biochemia struktur plemnika ssaka. Pages 99–124 *in* A. Łukaszczyk, B. Bilińska, J. Kawiak and Z. Bielańska-Osuchowska, eds. Ultrastruktura i funkcja komórki, mechanizmy regulujące spermatogenezę. Państwowe Wydawnictwo Naukowe PWN, Warszawa, Poland.

Sutovsky, P. and Manandhar, G. 2006. Mammalian spermatogenesis and sperm structure: anatomical and compartmental analysis. Pages 1–31 *in* Ch. De Jonge and Ch. Barratt, eds. The sperm cell. Production, maturation, fertilization, regeneration. Cambridge University Press, Cambridge, UK.

Swanson, E. W. and Bearden, H. J. 1951. An eosin-nigrosin stain for differentiating live and dead bovine spermatozoa. J. Anim. Sci. **10**: 981–987.

Szczygieł, M. and Kurpisz, M. 1996. Struktura plemnikowego DNA ssaków. Post. Biol. Kom. **23**: 211–219.

Veeramachaneni, D. N. R., Moeller, C. L. and Sawyer, H. R. 2006. Sperm morphology in stallions: Ultrastructure as a functional and diagnostic tool. Adv. Reprod. **22**: 683–692.

Ward, M. A. and Ward, W. S. 2004. A model for the function of sperm DNA degradation. Reprod. Fertil. Dev. **16**: 547–554.

Wenkoff, M. S. 1978. A sperm mid-piece defect of epididymal origin in two Hereford bulls. Theriogenology **10**: 275–281.

Wysokińska, A., Kondracki, S., Banaszewska, D. and Zajda, J. 2008. Cechy ejakulatu knurów inseminacyjnych w zależności od rasy i pory roku. Rocz. Nauk. PTZ **4**: 233–242.

Zalenskaya, I. A. and Zalensky, A. O. 2004. Non-random positioning of chromosomes in human sperm nuclei. Chromosome Res. **12**: 163–173.

Zambelli, D. and Cunto, M. 2006. Semen collection in cats: techniques and analysis. Theriogenology **5**: 59–65.